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A model employing low-cost, convenient, readily available tissue that consistently expresses a reproducible level of injury is required to screen for potential vesicant antagonists. We have developed a human whole blood, vesicant vapor exposure model. Blood obtained by venipuncture was diluted 40% with RPMI 1640 medium (Gibco/BRL, Grand Island, NY) and 5 ml aliquots dispensed into Costar trays (Allegience Health Care, Lee, MA). Half the samples were exposed for 12 min to 2-chloroethyl ethyl sulfide (CEES) vapor (1.5 mg/L/min), while the other half received carrier gas alone. Following such exposure, samples were incubated at 37°C, 5% CO2 for a total of 24 hr. Red blood cells were then lysed (Easy-Lyse™, Leinco Technologies, St. Louis, MO), and white blood cell viability was measured colorimetrically (ProCheck™ Cell Viability Assay, Intergen Co., Purchase, NY). CEES exposure decreased viability relative to controls (controls=100% viability). The viabilities for six CEES exposure trials (2 subjects, 3 times each) ranged from 44.3%-58.2%, with group means (n=3, by subject) of 52.6% and 48.7%, respectively. These group viabilities were not different from each other, but were significantly ($p<.05$) depressed compared to controls. This model induced a significant and reproducible level of CEES injury. It appears suitable for the rapid in vitro screening of combinations of potential vesicant antagonists, since the model can accommodate any buffer-soluble, antagonist combination up to a 40% blood displacement.

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A HUMAN WHOLE BLOOD MODEL FOR SCREENING POTENTIAL VESICANT ANTAGONISTS

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EXECUTIVE SUMMARY

A model employing low-cost, convenient, readily available tissue that consistently expresses a reproducible level of injury is required to screen for potential vesicant antagonists. We have developed a human whole blood, vesicant vapor exposure model. Blood obtained by venipuncture was diluted 40% with RPMI 1640 medium (Gibco/BRL, Grand Island, NY) and 5 ml aliquots dispensed into Costar trays (Allegience Health Care, Lee, MA). Half the samples were exposed for 12 min to 2-chloroethyl ethyl sulfide (CEES) vapor (1.5 mg/L/min), while the other half received carrier gas alone. Following such exposure, samples were incubated at 37°C, 5% CO_2 for a total of 24 hr. Red blood cells were then lysed (Easy-Lyse™, Leinco Technologies, St. Louis, MO), and white blood cell viability was measured colorimetrically (ProCheck™ Cell Viability Assay, Intergen Co., Purchase, NY). CEES exposure decreased viability relative to controls (controls=100% viability). The viabilities for six CEES exposure trials (2 subjects, 3 times each) ranged from 44.3%-58.2%, with group means (n=3, by subject) of 52.6% and 48.7%, respectively. These group viabilities were not different from each other, but were significantly ($p<.05$) depressed compared to controls. This model induced a significant and reproducible level of CEES injury. It appears suitable for the rapid *in vitro* screening of combinations of potential vesicant antagonists, since the model can accommodate any buffer-soluble, antagonist combination up to a 40% blood displacement.

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INTRODUCTION

Sulfur mustard (dichloroethyl sulfide; HD) is a strong alkylating agent that can induce injury similar to that incurred by exposure to ionizing radiation (7). These radiomimetic-like actions include direct lethal effects on leukocytes, bone marrow, and rapidly dividing cells. Such actions are likely attributable to the alkylation of DNA, which leads to cross-links and strand breaks in this macromolecule. Mustard exposure will also cause inhibition of glucose metabolism and lactate production, as well as depletion of ATP and nicotinamide adenine dinucleotide (NAD).

The extremely harmful and lethal effects of mustard agents preclude their study in human subjects. Therefore, various animal and tissue models have been utilized in their study. Among these diverse models has been the hairless guinea pig (8), the isolated perfused porcine skin flap (12), normal human epidermal keratinocytes and isolated peripheral blood lymphocytes (6), a constructed human dermal equivalent (5), and the hairless mouse or the mouse ear vesication model (4, 9, 10, 11). This laboratory has used artificial human skin models (1, 3) to explore several intracellular interventions as a means of providing vesicant protection. These interventions included preventing NAD depletion using niacinamide, inhibiting intracellular proteolytic enzyme activity with Leupeptin, or blocking calcium-mediated events with the calmodulin antagonist CGS-9343B. Each potential mustard antagonist was demonstrated to be nontoxic to the artificial human skin systems studied. Unfortunately, none appeared to offer significant protection from the detrimental effects of 2-Chloroethyl ethyl sulfide (CEES; half-mustard) exposure (2). However, since each antagonist was studied singly, this suggests blocking only one of the potential injury pathways is insufficient to prevent or reduce mustard-induced injury. Perhaps use of these intracellular mustard antagonists in combination with simultaneously blocking of the various injury pathways would be more effective than use of a single antagonist. A readily available tissue, like human whole blood (HWB), might be useful to rapidly screen for appropriate combinations of various antagonists that afford protection to CEES insult. The present study defined development of a HWB model in which levels of CEES-induced injury could be consistently reproduced.

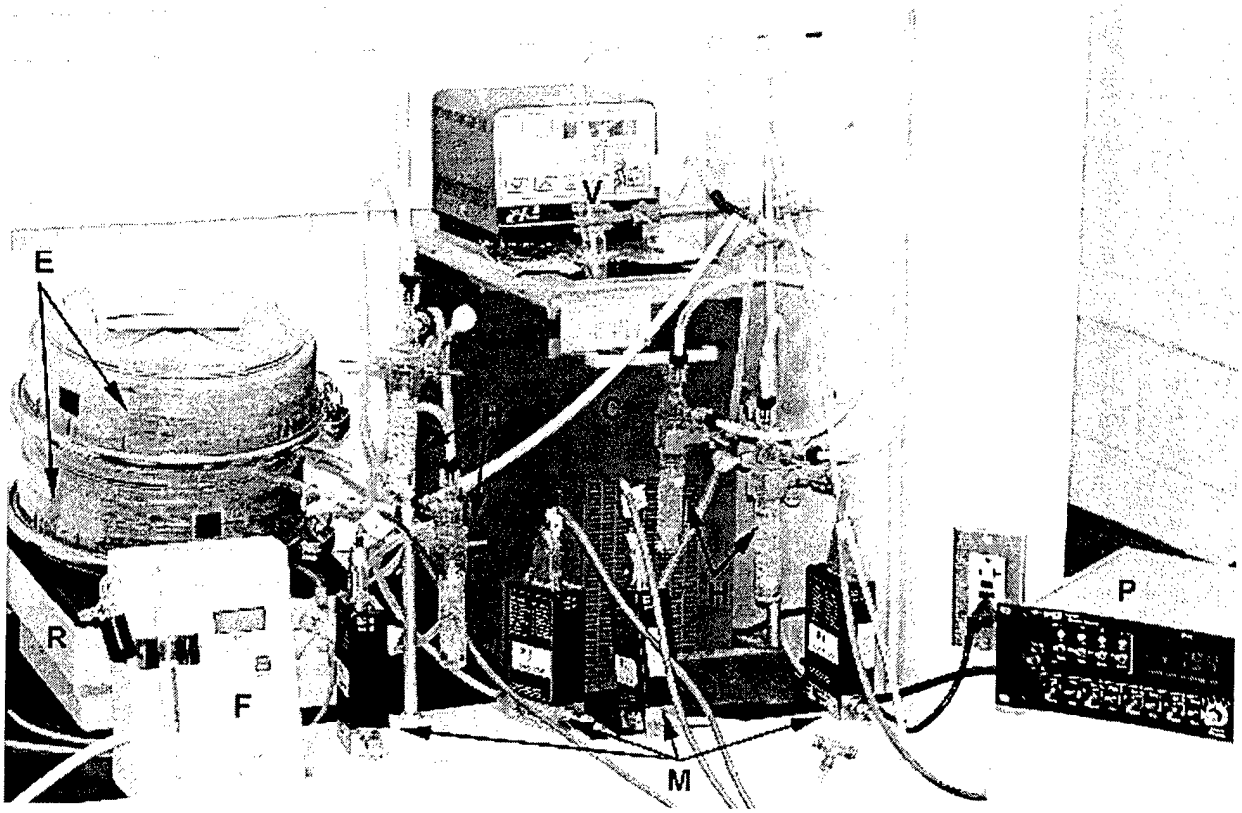
METHODS

Human Whole Blood (HWB) Collection/Dilution. HWB was obtained by venipuncture from two subjects on three separate occasions (6 testing days) in the following manner: Using a plastic 60 ml syringe (Becton Dickinson &Co., Rutherford, NJ) containing 140 µl heparin (Sigma Cat #H-4898, St Louis, MO; 4U/ml blood), 35 ml of HWB was drawn by venipuncture using a 21G "butterfly" needle (Terumo Medical Corp., Elkton, MD). A 40% dilution of HWB was made by combining 32 ml of heparinized HWB with 21.3 ml of RPMI medium (Gibco/BRL, Grand Island, NY).

Vesicant Exposure Procedure. After calibrating the gas flows of the exposure apparatus, 5 ml aliquots of diluted HWB were dispensed into 2, 6-well Costar trays (Allegience Health Care, Lee, MA), 5 samples per tray. One tray of samples was placed in each of the 2 chambers of the exposure apparatus (Figures 1, 2), which were mounted with Velcro™ to the top of a rotator (Caframo LTD, Ontario, Canada). One chamber received 12 min of humidified CEES vapor (1.5 mg/L/min; 18 mg total), while the other received only carrier gas (humidified air; L/min). During exposure the chambers were rotated at 15 revolutions per minute (rpm) to ensure blood homogeneity and uniform interaction with the respective treatment gases. The samples were removed from the chambers and allowed to "outgas" for 5 min and then incubated, with mixing, at 37°C, 5% CO_2 for a total of 24 hr.

Figure 1. Vapor Exposure Chamber and 6-Well Costar Tray (with simulated blood).

Figure 2. Vapor Exposure Apparatus



Vapor exposure apparatus setup including MKS type 247, 4-channel power supply (**P**; MKS, Andover, MA), MKS type 1179A mass flow controllers (**M**; MKS, Andover, MA) to regulate gas flow, Polystat™ constant temperature circulator (**C**; Cole Palmer, Vernon Hills, IL), vesicant containment vessel with bubbler (**V**), four humidifying bubblers (**H**), two exposure chambers (**E**), Type Reax 3 rotator (**R**; Caframo LTD, Ontario, Canada), and model 650 digital flowmeter (**F**; Humonics, Folsom, CA) to measure gas flow rates.

White Blood Cell (WBC) Isolation Procedure. The following procedure was performed to isolate a population of WBCs from HWB. From each well, 400 µl of blood was transferred to 15 ml polystyrene conical tubes. To each tube, 8 ml of red blood cell lysing solution (Easy-Lyse™, Leinco Technologies, St. Louis, MO) was added, immediately mixed, and incubated at room temperature (RT) for 10 min. The tubes were centrifuged at 1,300 rpm (300-500 g) for 5 min at RT; the supernatants were decanted, and then gently vortexed to resuspend the cells. The cells were then washed by adding 4 ml of Easy-Lyse™ buffer to each tube, mixed, and centrifuged as before, supernatants decanted, and cells resuspended. After a repeat of this washing procedure, the samples were reconstituted with 500 ml of RPMI 1640 medium (no phenol red) and mixed.

Viability Assay. Viability was measured colorimetrically using the ProCheck™ Cell Viability Assay (Intergen Co., Purchase, NY). The assay is based on the enzymatic conversion, by metabolically active cells, of the tetrazolium salt XTT from an oxidized tetrazole to a reduced formazan compound. The degree of color change from yellow to an orange/red is proportional to the relative number of living cells. The procedure was

as follows: Three, 100 μl aliquots of cell suspension (test required 10^4-10^6 cells/ml for optimal results after 4 hr incubation) were added to 3 wells of a 96-well plate. Each well then received 20 μl of ProCheck™ viability reagent. The plate was covered and incubated for 4 hr at 37°C, 5% CO_2. Following incubation, the plate was read at 480 nm on a Dynatech 7000 plate reader (Thermo Labsystems, Franklin, MA).

Cell Counts. WBC counts of the cell suspension were performed on a Coulter Z-1 particle counter (Beckman Coulter, Inc., Miami, FL) by mixing 200 μl of the WBC suspension with 10 ml of Coulter Z-1 diluent and counting the cells in the resulting mixture (cells > 4μ in size).

RESULTS

The mean (±SD) WBC counts, representing 51-fold dilutions of the WBC suspensions tested for viability, are shown in Table 1. Statistical comparisons of the WBC counts between the CEES-treated and their respective control groups demonstrated no significant ($p<.05$) differences for any of the six trials.

The viability data of all six trials had a range from 44.3% to 58.2%, with grand mean viabilities (n=3, by subject) of 52.6% and 48.7%, respectively (Table 2). These group viabilities were not different from each other, but were significantly ($p<.05$) depressed compared to controls.

Table 1: Mean White Blood Cell (WBC) Counts in Human Whole Blood Unexposed (Controls) or Exposed to 2-Chlorethyl Ethyl Sulfide (CEES)

Subject #	Trial #	Controls [Cells/ml]	CEES [Cells/ml]
	1	15,744 ± 3,006	12,438 ± 1,166
1	2	15,244 ± 3,548	12,634 ± 1,950
	3	12,174 ± 2,890	11,068 ± 1,502
	4	14,246 ± 3,760	13,466 ± 3,346
2	5	29,574 ± 5,674	24,172 ± 3,190
	6	17,832 ± 684	15,112 ± 2,304

WBC counts represent Means ± SD from 5 repeated measures (except trial 6 control which had 4 repeated measures).
Note: None of the CEES-treated cell counts were significantly ($p<.05$) different from their respective controls.

Table 2: White Blood Cell (WBC) Viability in Human Whole Blood Unexposed (Controls) or Exposed to 2-Chlorethyl Ethyl Sulfide (CEES)

Subject #	Trial #	Viability (%; CEES-treated vs. Control)
	1	45.9*
1	2	58.2*
	3	53.0
		Grand Mean = 52.6% (n=15)**
	4	44.3*
2	5	49.4*
	6	52.0*
		Grand Mean = 48.7% (n=15)**

WBC % viabilities represent means determined from 5 repeated measures for each trial. * Significantly different ($p<.05$) from unexposed controls. ** Grand Means, though significantly depressed relative to controls, were not different from each other.

DISCUSSION

In order for an experimental model to be useful as a screening tool in vesicant research, it must be able to induce both a significant and reproducible injury. It should also be low in cost and convenient. If not, it offers little advantage over more complex established models. Additionally, the tissue used in the model should be relevant with respect to vesicant injury.

HWB obtained by venipuncture was convenient, readily available, and inexpensive. It also represented a tissue normally affected by vesicant exposure of the skin or lungs. Isolating WBCs from HWB and measuring their viability was a rapid process. Viability served as an easily assayable injury index to evaluate the level of CEES injury. The results seen here suggested the HWB vesicant vapor exposure model has the appropriate attributes to support primary screening of potential vesicant antagonists.

With the HWB vesicant vapor exposure model, it was demonstrated that 12-min CEES vapor exposures (1.5 mg/L/min; 18 mg total dose) with a HWB rotational rate of 15 rpm reduced the WBC viability of both subjects by about 50% (52.6% and 48.7%, respectively). Vesicant exposures were relatively easy to perform, and the degree of injury was reproducible by controlling the length of vesicant vapor exposure and HWB rotational rate. These decrements in viability were significant when compared to their respective controls, but were not different from each other. This suggested the methods and procedures employed could consistently induce a similar level of injury. Moreover, the viability decrement of ~50% would likely not be so severe as to preclude demonstration of a protective effect by a vesicant antagonist.

Thus, this model could be employed to identify the effectiveness of various combinations of potential vesicant antagonists. Only those antagonists or combinations that showed efficacy in this model could then be tested in more advanced tissue constructs or animal models, thereby conserving research resources.

CONCLUSIONS

The HWB vesicant vapor exposure model employed a readily available, low-cost tissue source, induced a reproducible level of injury, and was designed to permit vesicant antagonist combinations of up to a 40% blood displacement. Such a model appears suitable for the rapid *in vitro* screening of combinations of potential vesicant antagonists.

RECOMMENDATIONS

The use of the HWB vesicant vapor exposure model is recommended to rapidly assess the efficacy potential of vesicant antagonist combinations. Such a model could play an important role in identifying those antagonists worthy of further study. This approach would reduce pressure on limited budget resources and decrease the use of animals in the Chemical Defense Research Program.

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